The Novel Cyclin-Dependent Kinase 4/6 Inhibitor Ribociclib (LEE011) Alone and in Dual-Targeting Approaches Demonstrates Antitumoral Efficacy in Neuroendocrine Tumors in vitro

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Abstract: BACKGROUND/AIM Cyclin-dependent kinases (CDKs) are crucial for cell cycle regulation, and alterations in the cell cycle are often observed in human cancer. CDK4/6 in particular orchestrates G1 phase progression and the G1/S transition. Here, we investigated the in vitro effects of the CDK4/6 inhibitor LEE011 in human neuroendocrine tumor cells. METHODS The human neuroendocrine tumor cell lines BON1, QGP1, NCI-H727 and GOT1 were treated with different concentrations of LEE011 alone and in combination with 5-fluorouracil and everolimus. RESULTS Cell viability decreased in a time- and dose-dependent manner in BON1, QGP1, and NCI-H727 cells upon LEE011 treatment, whereas GOT1 cells were treatment resistant. Treatment sensitivity towards LEE011 was associated with the high expression of cyclin D1 and Rb. LEE011 caused the dephosphorylation of Rb and a subsequent G1 phase cell cycle arrest. Combined treatment with LEE011 and 5-fluorouracil or everolimus showed a significant enhancement in the inhibition of cell viability when compared to single-substance treatments due to PI3K-Akt-mTOR and Ras-Raf-MEK-ERK pathway downregulation and cooperative downregulation of cell cycle components. However, LEE011 also exhibited antagonizing effects with 5-fluorouracil, protecting NET cells from DNA-damaging chemotherapy by blocking PARP cleavage and caspase-3/7 activity. CONCLUSIONS Our data demonstrate that the CDK 4/6 inhibitor LEE011 exhibits promising antitumoral properties alone and in combination treatment approaches with 5-fluorouracil or everolimus in human neuroendocrine tumor cell lines.

DOI: https://doi.org/10.1159/000463386

Original Paper

Neuro
-endocrinology

Neuroendocrinology 2018;106:58–73
DOI: 10.1159/000463386

Received: October 10, 2016
Accepted after revision: February 11, 2017
Published online: February 23, 2017

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Keywords
CDK4/6 inhibitor · Ribociclib · LEE011 · Neuroendocrine tumor · Dual targeting

Abstract
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Introduction

Gastroenteropancreatic neuroendocrine tumors (GEP-NETs) are the second most common gastrointestinal malignancy after colorectal cancer [1]. Due to the variable cellular and tumor phenotypes of GEP-NETs, the clinical presentation is nonspecific; hence, the diag-

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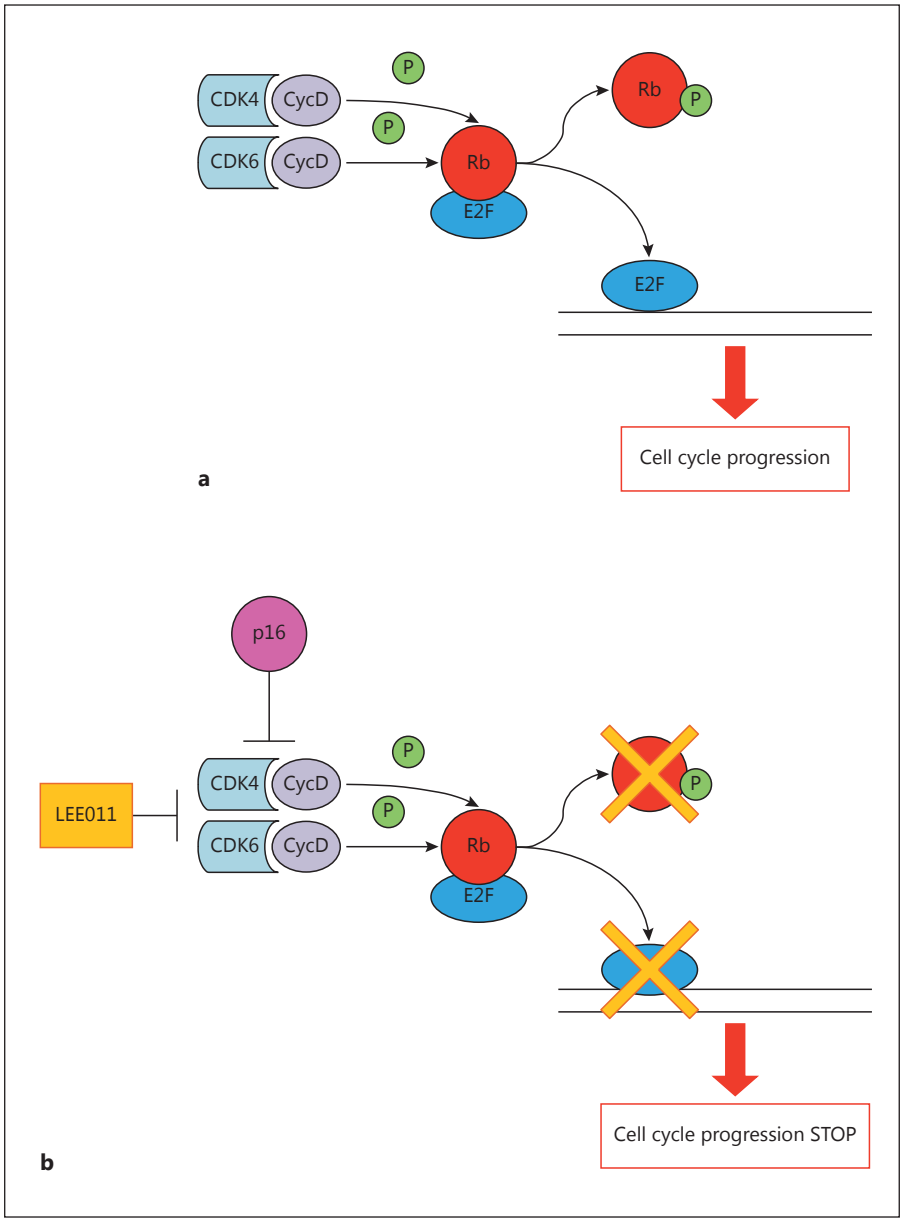

Fig. 1. Proposed and simplified mode of action of the CDK4/6 inhibitor LEE011 on the cell cycle. **a** Activated cyclinD-CDK4/6-Rb axis leads to G1/S cell cycle progression via the phosphorylation of Rb and subsequent activation of the transcription factor E2F. **b** Blocking the cyclinD-CDK4/6-Rb axis leads to G1 phase cell cycle arrest through either the endogenous CDK4/6 inhibitor p16 or the small molecule CDK4/6 inhibitor LEE011.

nosis is often at a late and an advanced metastatic state [1, 2]. The paucity of successful targeting agents for GEP-NETs is mostly due to the complexity and the rarity of GEP-NETs, as well as the intrinsic differences in malignant potential because of their heterogeneity and the dissimilar clinical presentation [1, 3]. Current therapeutic approaches for GEP-NETs, such as biotherapy, molecular targeted therapy, chemotherapy, and peptide receptor radionuclide therapy are limited in their effectiveness; ergo, new strategies are urgently needed [4]. The cyclin-dependent kinases (CDKs) are the main regulators of the cell cycle transition and catalyze the phosphorylation of key proteins and transcription factors, and the aberrant expression of these CDKs due to gene mutation, amplification or overexpression often leads to cancer cell formation [5]. In addition to CDKs, their associated substrates (cyclins) are expressed in a spatiotemporal-dependent manner, enabling a controlled step-by-step cell cycle progression [6]. In particular, the complexes cyclinD-CDK4 and cyclinD-CDK6 regulate the G0–G1 transition in quiescent cells and the early G1 phase transition in proliferating cells by phosphorylating the tumor suppressor reti-

Neuroendocrinology 2018;106:58–73
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noblastoma protein (Rb) and thus activating the transcription factor E2F [7]. Activated E2F regulates transcription through G1/S phase by coordinating gene transcription of cell cycle progression-relevant proteins [8] (Fig. 1a). The endogenous tumor suppressor p16INK4a (p16) blocks CDK4/6 and causes a permanent G1 phase arrest [9]. Hence, the CDK4/6 inhibitor LEE011 (Novartis, Basel) offers a very promising molecular targeting approach by selectively downregulating the proliferation-associated cyclinD-CDK4/6-Rb axis (Fig. 1b). In various studies, the small molecule CDK4/6 inhibitor LEE011 has been shown to have antiproliferative characteristics with only weak side effects in comparison to previous nonselective CDK inhibitors [10]. Currently, a clinical phase 2 trial with LEE011 is recruiting patients with advanced NETs of foregut origin (NCT02420691). Therefore, the phylogenetic homologues CDK4 and CDK6 [11] represent promising novel molecular targets for therapeutic NET treatments. Here, we investigate the effects of the CDK4/6 inhibitor LEE011 on different NET cell lines in vitro.

Materials and Methods

Materials

Ribociclib (LEE011) and everolimus (Rad001) were provided by Novartis (Basel, Switzerland). 5-Fluorouracil (5-FU) was purchased from Selleckchem (Houston, TX, USA). Everolimus and 5-FU were diluted in dimethyl sulfoxide (DMSO; 10 mM stock solution; Sigma, D8418). LEE011 was diluted in deionized water. Dulbecco's Modified Eagle's Medium – NutrientMixture F-12 (1:1) (DMEM/F12) and penicillin/streptomycin were obtained from Gibco/Invitrogen (Karlsruhe, Germany). RPMI medium (with L-glutamine, $NaCO_3$) and phosphate-buffered saline (PBS) were purchased from Sigma, whereas trypsin-EDTA (10×) was acquired from PAA Laboratories (Cölbe, Germany). Fetal bovine serum (FBS) and amphotericin B were acquired from Biochrom (Berlin, Germany).

Cell Culture

The human pancreatic NET (pNET) cell line BON1 [12] (kindly provided by Prof. R. Göke, Marburg, Germany) and the pancreatic islet tumor cell line QGP1 (acquired from JCRB Cell Bank [Japanese Collection of Research Bioresources Cell Bank]) [13] were grown in DMEM/F12 (1:1) supplemented with 10% FBS, 1% penicillin/streptomycin and 0.4% amphotericin B. Human bronchopulmonary neuroendocrine NCI-H727 (in all figures named H727) tumor cells [14] (acquired from ATCC, Manassas, VA, USA) and human midgut carcinoid GOT1 cells [15] (kindly provided by Prof. O. Nilsson, Sahlgrenska University Hospital, Göteborg, Sweden) were grown in RPMI medium supplemented with 10% FBS, 1% penicillin/streptomycin and 0.4% amphotericin B. The GOT1 culture medium was additionally supplemented with 5 µg/mL apo-transferrin and 0.135 IU/mL insulin. All human neuroendocrine cell lines were received and cultured as described previously [16, 17]. The cells were tested and determined to be mycoplasma free and incubated at 37°C and 5% CO_2.

Cell Viability Assessment

Cells were counted (Cellcounter Countess, Invitrogen, Germany), seeded, and grown for 24 h in 96-well plates at densities of 1,500 (BON1), 2,000 (QGP1 and NCI-H727), and 30,000 (GOT1) cells per well. After 24 h, the cells were treated in 10% FBS with different concentrations of LEE011 alone and in combination with 10 nM everolimus or 5 µM 5-FU, as previous studies had shown significant effects for the respective substance concentrations in NET cells in vitro [16, 18]. Metabolic activity was measured with a CellTiter 96® Aq_{ueous} One Solution cell viability assay (Promega, Madison, WI, USA) after 72 and 144 h of incubation. Then, the treated cells were incubated for 4 h with CellTiter 96 solution, and the absorbance was determined at 490 nm using an ELISA plate reader (Orion II; Berthold Detection Systems, Pforzheim, Germany).

Cell Cycle Analysis by Flow Cytometric Analysis

Cell cycle distribution was analyzed following the quick method from Nature Protocols "Analysis of Apoptosis by Propidium Iodide Staining and Flow Cytometry" [19] (BD Accuri C6 Analysis). Cells were cultured in 6-well plates (4×10^5 BON1 cells/well and 5×10^5 QGP1 and NCI-H727 cells/well) for 24 h in complete medium. After 24 h, the medium was replaced with fresh 10% FBS medium and incubated with 10 µM LEE011 alone or in combination with 5 µM 5-FU or 10 nM everolimus. After 72 h, the cells were washed with PBS and treated with 300 µL of trypsin at 37°C for 5 min. Cells were collected and centrifuged at 2,000 rpm for 5 min. After another wash cycle with PBS, the cells were centrifuged again. The pellets were resuspended in 350 µL of propidium iodide, and 8 h later, 20,000 events from each sample were analyzed.

Protein Extraction and Western Blotting

For Western blot experiments, 450,000 (BON1) and 600,000 (QGP1 and NCI-H727) cells were seeded in 10-cm plates and grown for 24 h in complete medium. Then, the medium was replaced with fresh 10% FBS medium, and cells were incubated with different concentrations of LEE011 (500 nM and 10 µM) alone or in combination with 5-FU (5 µM) and everolimus (10 nM). The incubation times proceeded for up to 72 h. After incubation, the cells were washed twice in cold PBS on ice and lysed in 500 µL of lysis buffer (M-PER Mammalian Protein Extraction Reagent containing HALT protease and phosphatase inhibitor cocktail; Thermo Scientific, Rockford, IL, USA). Lysates were centrifuged at 13,000 rpm for 10 min. Supernatants were adjusted to the same protein concentration (30–50 µg/50 µL) (Rotiquant Universal; Carl Roth, Karlsruhe, Germany) and denatured in sodium dodecyl sulfate (SDS) sample buffer (0.25% Tris HCL, 40% glycerol, 2% SDS, 1% dithiothreitol, and bromophenol blue, pH 8.8). Equal amounts of protein were separated on an SDS polyacrylamide gel and electrotransferred for 60 min onto PVDF membranes (Immobilone; Millipore, Eschborn, Germany) using a semi-dry Western blot technique. After blocking in 2% skimmed milk powder, the membranes were incubated overnight at 4°C in appropriate dilutions of primary antibodies against pAKT (Ser473) (#4060), AKT (#2920), pERK1/2 (Thr202/Tyr204) (#4370), p4EBP1 (Ser65) (#9451), 4EBP1 (#9644), pRb (Ser780) (#9307), pCDK1 (Tyr15) (#4539), CDK1 (#9116), cyclin B1 (#12231), cyclin D1 (# 2926),

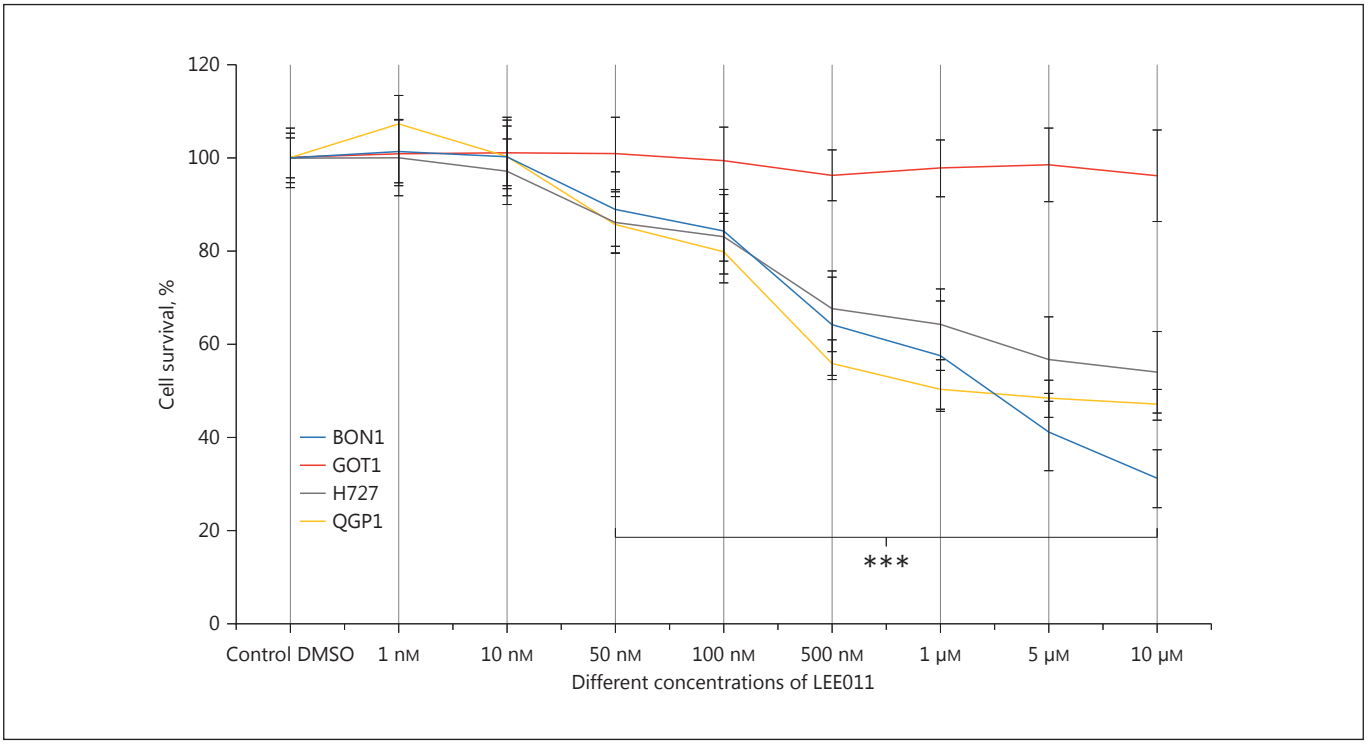

Fig. 2. The effect of different concentrations of LEE011 on cell survival of 4 different NET cell lines after 144 h of incubation. Human neuroendocrine pancreatic BON1, pancreatic islet QGP1, bronchopulmonary H727, and midgut GOT1 cells were incubated with LEE011 in a concentration range of 1 nM to 10 µM for 144 h. The calculated means and standard deviation of at least 3 independent experiments are shown. Statistically significant differences in the results in comparison to control cells treated with DMSO 50 nM to 10 µM are represented by *** $p < 0.001$. Data are presented as mean ± SD.

cyclin D3 (#2936), CDK4 (#12790), CDK6 (#13331), Chk1 (#2360), pChk2 (Ser19) (#2666), pChk2 (Thr68) (#6334), Chk2 (#6334), Parp (#9542), PCNA (#2586) (all from Cell Signaling Technology, Danvers, MA, USA), p16 INK4A (ab151303) (Abcam, Cambridge, UK), Rb (#614602) (Biolegend, San Diego, CA, USA), actin (A5441) (Sigma, St. Louis, CA, USA), and ERK1/2 (06-182) (Merck-Millipore, Darmstadt, Germany). After washing in TBS, the membranes were incubated with a peroxidase-conjugated secondary antibody (1:25,000) for 2 h. The blots were washed and immersed in the chemiluminescent substrate Super Signal West Dura (Thermo-Scientific), and images were taken with an ECL Chemocam Imager (INTAS, Göttingen, Germany).

BON1	QGP1	H727
IC_{50} = 2.6 µM	IC_{50} = 1.2 µM	IC_{50} = 10.9 µM

Fig. 3. The 50% inhibitory concentration (IC_{50}) of LEE011 in 3 different NET cell lines after 144 h of incubation.

Caspase-3/7 Activity Assay

To measure the apoptotic activity, we used the Apo-One homogeneous caspase-3/7 assay kit (Promega, G7790). Therefore, we seeded 10,000 cells per well of each cell line and incubated for 72 h in different concentrations of LEE011 alone or in combination with 5 µM 5-FU and 10 nM everolimus. Cells were incubated for 72 h, and caspase-3/7 activity was assessed following the manufacturer's instructions.

Statistical Analysis

The results are displayed as the mean ± standard deviation of the mean (SD) of at least 3 independently performed experiments. Each cell viability experiment consisted of at least 6 samples per substance concentration and incubation period. A priori tests considering the normal distribution and homogeneity of variances were performed applying the Kolmogorov-Smirnov test and Levene's test using the SPSS statistical package (version 13.0 for Windows, SPSS Inc., Chicago, IL, USA). When parametric criteria were met, an ANOVA comparison of means with a post hoc Tukey test or a 2-tailed *t* test was performed; when nonparametric criteria were met, a Kruskal-Wallis test was performed followed by a Mann-Whitney test. Statistical significance was assessed at $p < 0.05$.

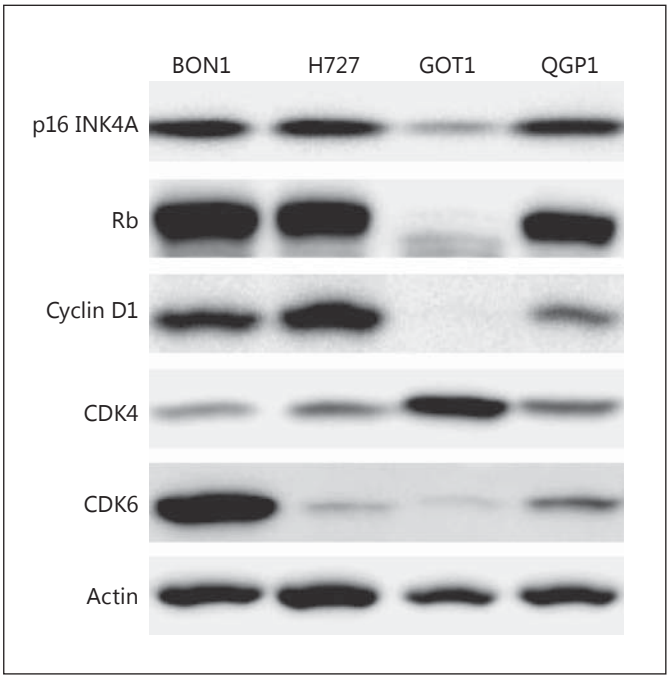

Fig. 4. Basal expression levels of proteins from the cyclinD-CDK4/6-Rb axis in all 4 NET cell lines. The expression of p16, Rb, cyclin D1 CDK4, and CDK6 was evaluated by Western blot analysis. A representative blot out of 3 independently performed experiments is shown.

Results

LEE011 Inhibits Cellular Proliferation in Three out of Four NET Cell Lines

Neuroendocrine pancreatic BON1 cells, pancreatic islet QGP1 NET cells and bronchopulmonary NCI-H727 NET cells showed significant treatment susceptibility in a time- and dose-dependent manner (Fig. 2). GOT1 cells showed treatment resistance at all concentrations and incubation times tested. All 4 cell lines were incubated with LEE011 at a concentration range of 1 nM to 10 μM. The most notable results were obtained after 144 h of incubation with LEE011, where significant differences in all 3 cell lines in comparison to the Control DMSO-treated cells were reached with a concentration of only 50 nM. At a maximum concentration of 10 μM, cell viability decreased in BON1 cells to 31.08 ± 6.13%, in QGP1 cells to 47 ± 3.28%, and in NCI-H727 cells to 53.90 ± 8.74%. In QGP1 cells, an efficacy limit with a plateau effect was reached at a concentration of 1 μM. Further concentration augmentation did not yield a significant survival decrease enhancement in QGP1 cells. The most sensitive cells towards the treatment, following the IC_{50} values of each cell line after 144 h of incubation with LEE011 (1 nM to 10 μM), were QGP1 (IC_{50} = 1.2 μM), followed by BON1 cells (IC_{50} = 2.6 μM) and NCI-H727 (IC_{50} = 10.9 μM) (Fig. 3).

Sensitivity to LEE011 Treatment Depends on Rb and Cyclin D1 Expression

Western blot analysis showed different baseline expression patterns of the cyclinD-CDK4/6-Rb axis in all 4 NET cell lines (Fig. 4). The tumor suppressor p16, Rb and cyclin D1 were stably expressed in neuroendocrine pancreatic BON1, pancreatic islet QGP1 and bronchopulmonary NCI-H727 cells, whereas they were poorly expressed in GOT1 cells. CDK4 was very strongly expressed in GOT1 cells and less strongly expressed but present in all others. BON1 cells showed the highest CDK6 expression, followed by QGP1, NCI-H727, and GOT1 cells.

LEE011 Causes G1 Phase Cell Cycle Arrest

In all 3 NET cell lines, flow cytometric analysis demonstrated that LEE011 caused a dose-dependent increase in G1 phase cell cycle arrest after incubation with cells for 72 h (Fig. 5). In BON1 (Fig. 5a) and QGP1 (Fig. 5b) cells, a significant increase in G1 phase cell cycle arrest was observed with concentrations as low as 1 μM and observed in NCI-H727 (Fig. 5c) cells with concentrations as low as 100 nM. At a maximum concentration of 10 μM, the mean G1 phase percent of BON1 (Fig. 5a), QGP1 (Fig. 5b), and NCI-H727 (Fig. 5c) cells increased to 77.5 ± 2.1, 89.9 ± 0.9, and 84.4 ± 4.9%, respectively. Consequently, the mean percent of cells in the G2 and S cell cycle phase significantly decreased in all 3 cell lines (Fig. 5).

Combined Treatment with LEE011, 5-FU and Everolimus Showed More Efficacy Than Single-Substance Treatment in Decreasing NET Cell Survival

For all 3 NET cell lines tested, the combined treatment led to significantly more effective results in mediating cell survival than single-substance treatment (Fig. 6). At all incubation times and in all 3 cell lines, the combined treatment with LEE011 (500 nM) and everolimus (10 nM) showed significantly higher decreases in cell survival than each single-substance treatment (Fig. 6). The combined treatment with 5-FU was more effective only at some of the tested incubation times and in some of the cell lines (Fig. 6). In BON1 cells, the combined treatment with 5-FU (5 μM) was only more effective after 72 h of

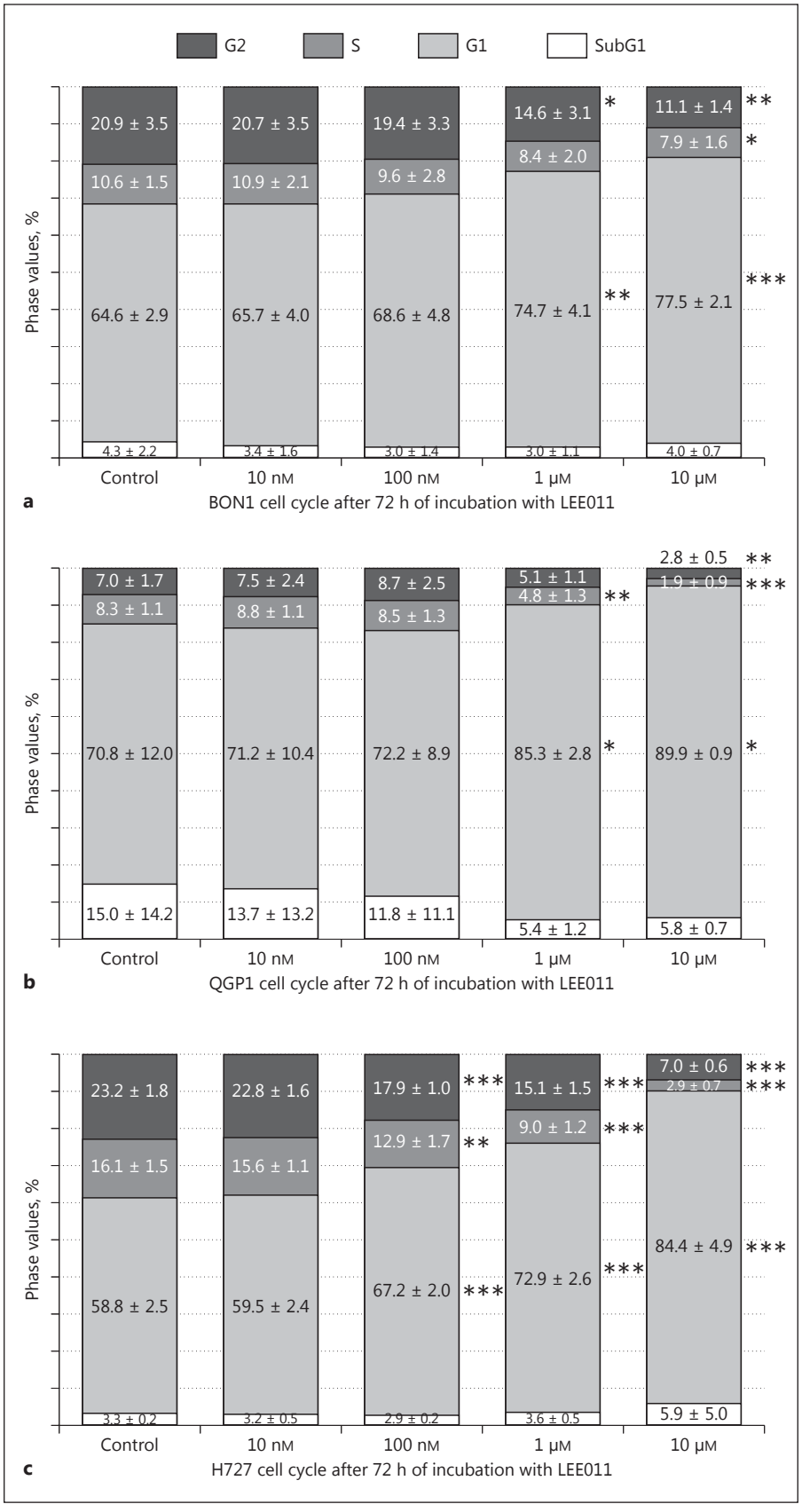

Fig. 5. Cell cycle analysis of BON1 (**a**), QGP1 (**b**), and H727 (**c**) cells with different concentrations of LEE011 (10 nM to 10 μM) after 72 h of incubation measured by flow cytometric analysis. The calculated means and standard deviation of at least 3 independent experiments are shown. Statistically significant differences in results in comparison to control cells treated with 10 nM to 10 μM DMSO are represented by * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Data are presented as mean ± SD.

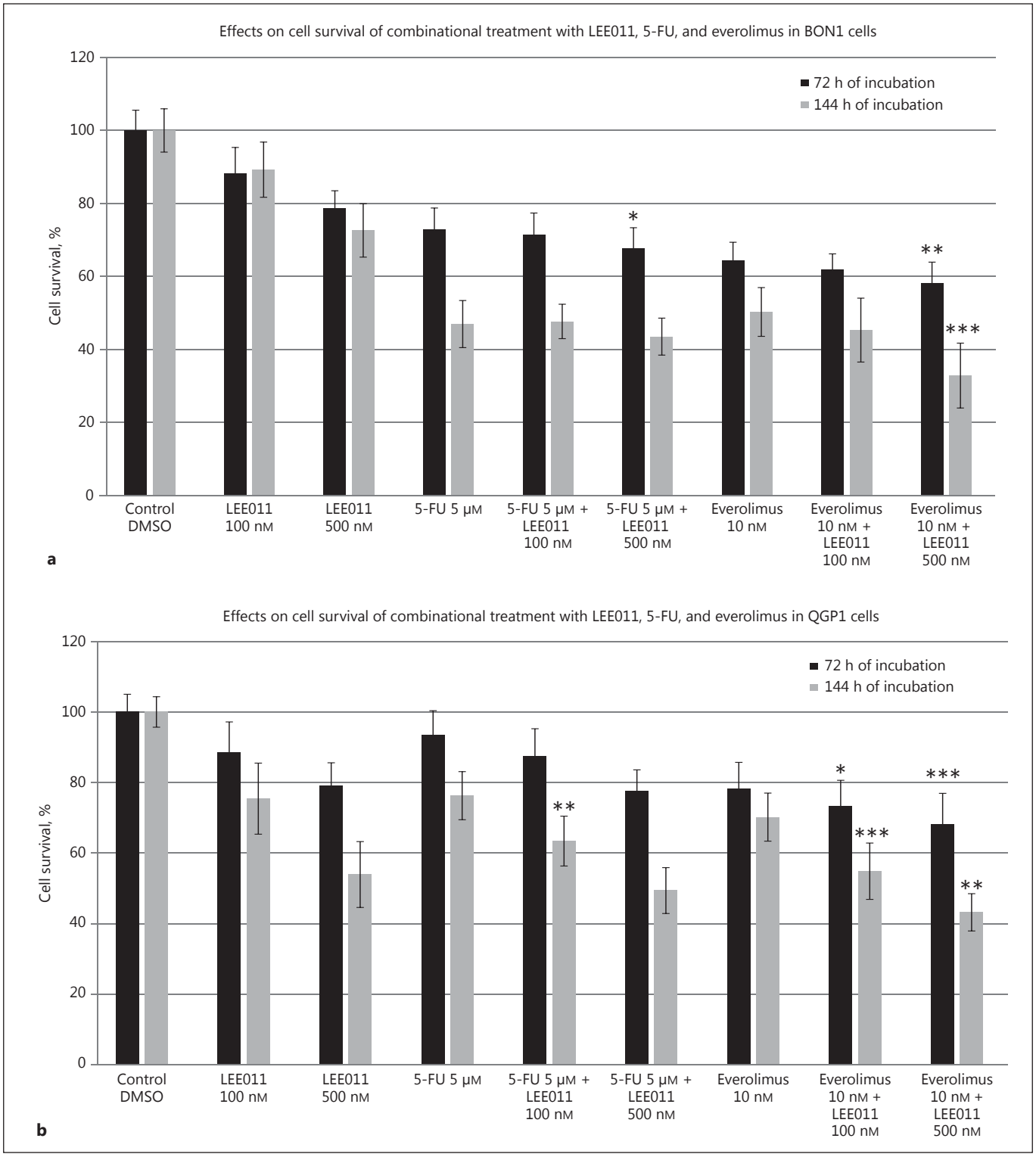

Fig. 6. Effect of LEE011 on cell survival of 4 different NET cell lines after 72 and 144 h of incubation. Human neuroendocrine pancreatic BON1 (**a**) and pancreatic islet QGP1 (**b**), and bronchopulmonary NCI-H727 (**c**) cells were incubated with LEE011 (100 and 500 nM) alone and in combination with 5-FU (5 μM) and everolimus (10 nM) for 72 and 144 h. The calculated means and standard deviation of at least 3 independent experiments are shown. Statistically significant differences in the results in comparison to single-substance treatments are shown; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are presented as mean ± SD.

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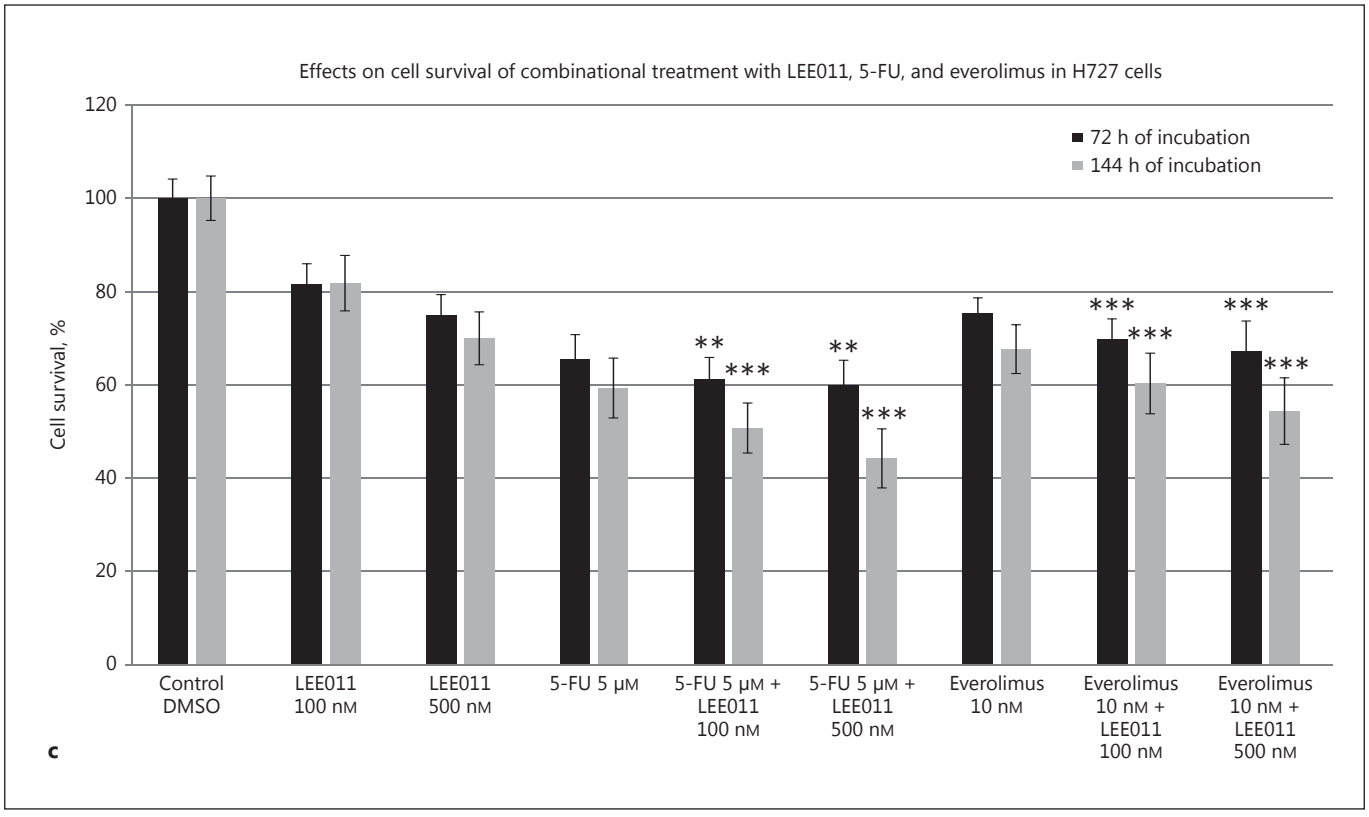

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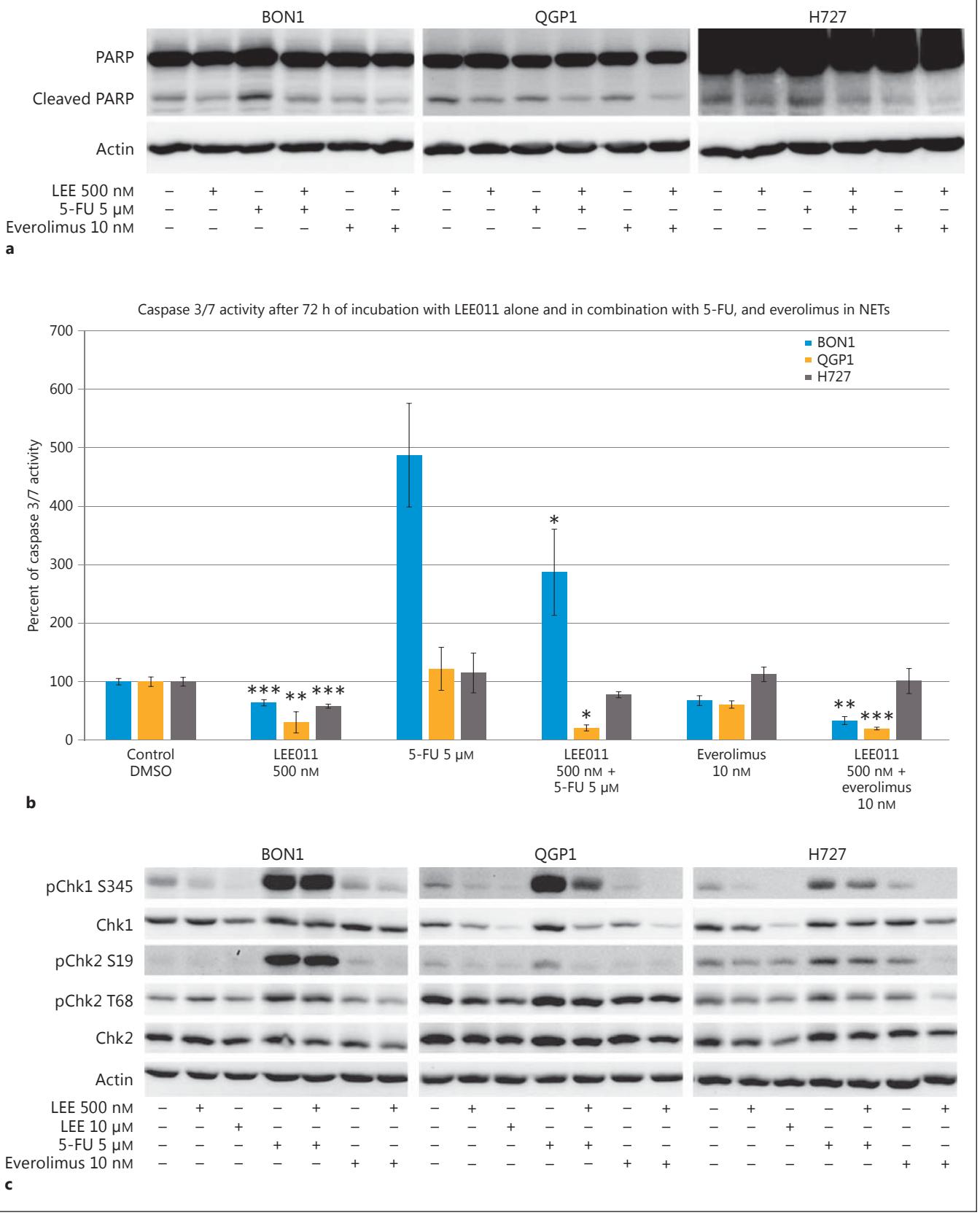
incubation with LEE011 (500 nM), and cell survival decreased to 67.62 ± 5.73% (Fig. 6a). The combined treatment with LEE011 (500 nM) and everolimus (10 nM) in BON1 cells showed significantly enhanced antiproliferative effects after 72 and 144 h, with a reduced mean cell survival of 58.07 ± 5.85 and 32.83 ± 8.89%, respectively (Fig. 6a). Furthermore, in QGP1 cells, the combined treatment with LEE011 (100 and 500 nM) and everolimus (10 nM) was more effective than single-substance treatment at both incubation times (72 and 144 h), and cell survival decreased to 73.33 ± 7.29 and 68.23 ± 8.70% after 72 h of incubation and 54.85 ± 7.98 and 43.21 ± 5.29% after 144 h of incubation, respectively (Fig. 6b). Whereas the combined treatment with 5-FU in QGP1 cells was only more effective at an incubation time of 144 h and LEE011 concentration of 100 nM, with a mean survival of 63.40 ± 7.06% (Fig. 6b). Only in bronchopulmonary NCI-H727 cells was the combination treatment with 5-FU more efficient than single-substance treatment at both incubation times and both concentrations of LEE011, with a mean survival of 61.28 ± 4.59% (5-FU 5 μM + LEE011 100 nM) and 59.94 ± 5.33% (5-FU 5 μM + LEE011 500 nM) after 72 h and 50.72 ± 5.36% (5-FU 5 μM + LEE011 100 nM) and 44.21 ± 6.34% (5-FU 5 μM + LEE011 500 nM) after 144 h of incubation (Fig. 6c). Again, in NCI-H727 cells, the combined treatment with everolimus showed significantly better results than the respective single-substance treatments, with a mean cell survival of 69.80 ± 4.33% (everolimus 10 nM + LEE011 100 nM) and 67.21 ± 6.46% (everolimus 10 nM + LEE011 500 nM) after 72 h of incubation and 60.28 ± 6.51% (everolimus 10 nM + LEE011 100 nM) and 54.35 ± 7.14% (everolimus 10 nM + LEE011 500 nM) after 144 h of incubation (Fig. 6c).

LEE011 Blocks the Apoptotic Cell Death Mechanism through PARP and Caspase-3/7 Cleavage and Chk1 Downregulation

Western blot analysis demonstrated the expression of PARP cleavage in human neuroendocrine pancreatic BON1, pancreatic islet QGP1 and bronchopulmonary NCI-H727 tumor cells when stimulated for 72 h with LEE011 (500 nM) alone and in combination with 5-FU (5 μM) and everolimus (10 nM) (Fig. 7a). In all 3 cell lines, LEE011 alone decreased PARP cleavage, whereas 5-FU induced PARP cleavage in comparison to control cells treated with DMSO. Everolimus alone induced only low levels of PARP cleavage. LEE011 in combination with



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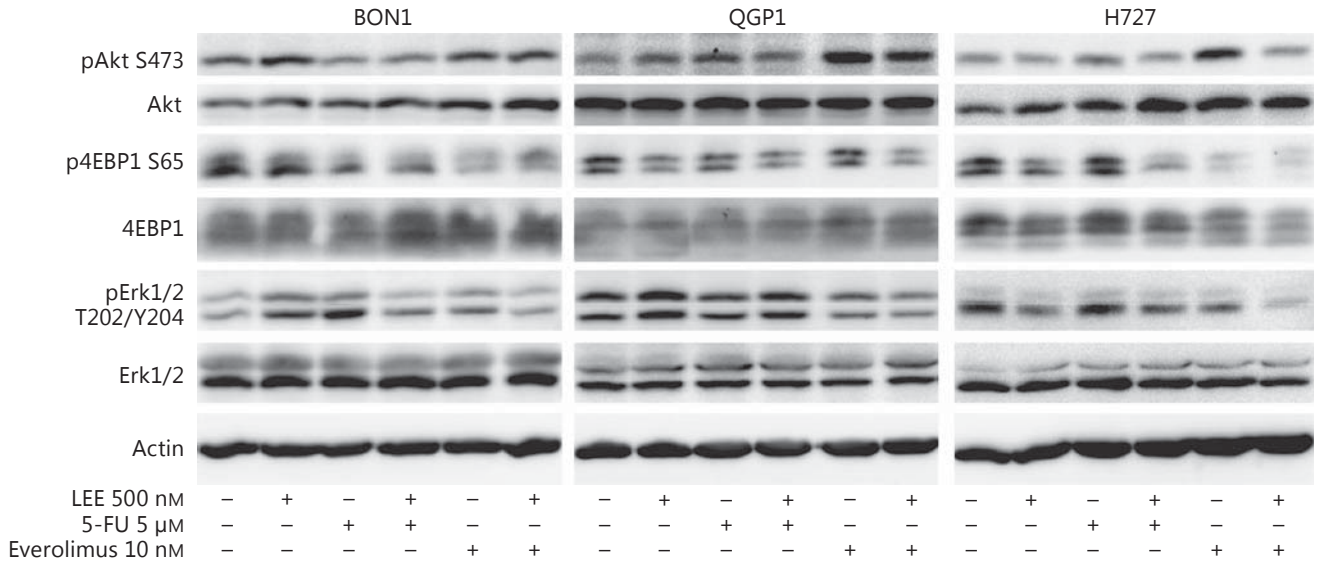

Fig. 8. The effects of LEE011 (500 nM) alone and in combination with 5-FU (5 μM) and everolimus (10 nM) on components of the PI3K-Akt-mTOR and Ras-Raf-MEK-ERK signalling pathway in BON1, QGP1, and H727 cells after 72 h of incubation analyzed via Western blot. A representative blot from 3 independently performed experiments is shown.

5-FU antagonizes the PARP-cleaving effects of 5-FU in all 3 cell lines. Furthermore, the weak PARP-cleaving effects of everolimus are also counteracted by LEE011. Mean caspase-3/7 activity was significantly decreased in all 3 cell lines after 72 h of incubation with LEE011 (500 nM) (Fig. 7b). Again, LEE011 in combination with 5-FU was shown to significantly counteract the induced caspase-3/7 activity of 5-FU in BON1 and QGP1 cells (Fig. 7b). Everolimus alone did not induce caspase-3/7 activity, and the combination treatment with LEE011 and everolimus significantly lowered the already low caspase-3/7 activity in BON1 and QGP1 cells (Fig. 7b). LEE011 treatment alone and in combination with 5-FU and everolimus lowered the expression and phosphorylation of Chk1/2 in all 3 NET cell lines analyzed (Fig. 7c).

Fig. 7. The effects of LEE011 (500 nM) alone and in combination with 5-FU (5 μM) and everolimus (10 nM) on caspase-3/7 activity and PARP cleavage after 72 h of incubation. **a** Western blot analysis of PARP cleavage in NETs. A representative blot from 3 independently performed experiments is shown. **b** Caspase-3/7 activity in BON1, QGP1, and H727 cells. The calculated means and standard deviation of at least 3 independent experiments are shown. Statistically significant differences in the results in comparison to single-substance treatments are shown; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are presented as mean ± SD. **c** Western blot analysis of Chk1 and Chk2 expression and phosphorylation in BON1, QGP1, and H727 cells. A representative blot from 3 independently performed experiments is shown.

Combined Treatment with LEE011 and 5-FU or Everolimus Downregulates Two Major Proliferative Signaling Pathways in NETs: the PI3K-Akt-mTOR Pathway and Ras-Raf-MEK-ERK Pathway

Western blot analysis demonstrated the expression of PI3K-Akt-mTOR and Ras-Raf-MEK-ERK pathway components in human neuroendocrine pancreatic BON1, pancreatic islet QGP1 and bronchopulmonary NCI-H727 tumor cells when stimulated for 72 h with LEE011 (500 nM) alone and in combination with 5-FU (5 μM) or everolimus (10 nM) (Fig. 8). In BON1 and QGP1 cells, treatment with LEE011 alone phosphorylates Akt and Erk but dephosphorylates 4EBP1. In NCI-H727 cells, LEE011 alone has no effect on Akt phosphorylation, but p4EBP1 and pErk are downregulated. In all 3 cell lines, treatment with LEE011, 5-FU or everolimus resulted in unfavorable proproliferative phosphorylation of each component analyzed, but the combination treatment with 5-FU and LEE011 or everolimus and LEE011 an-

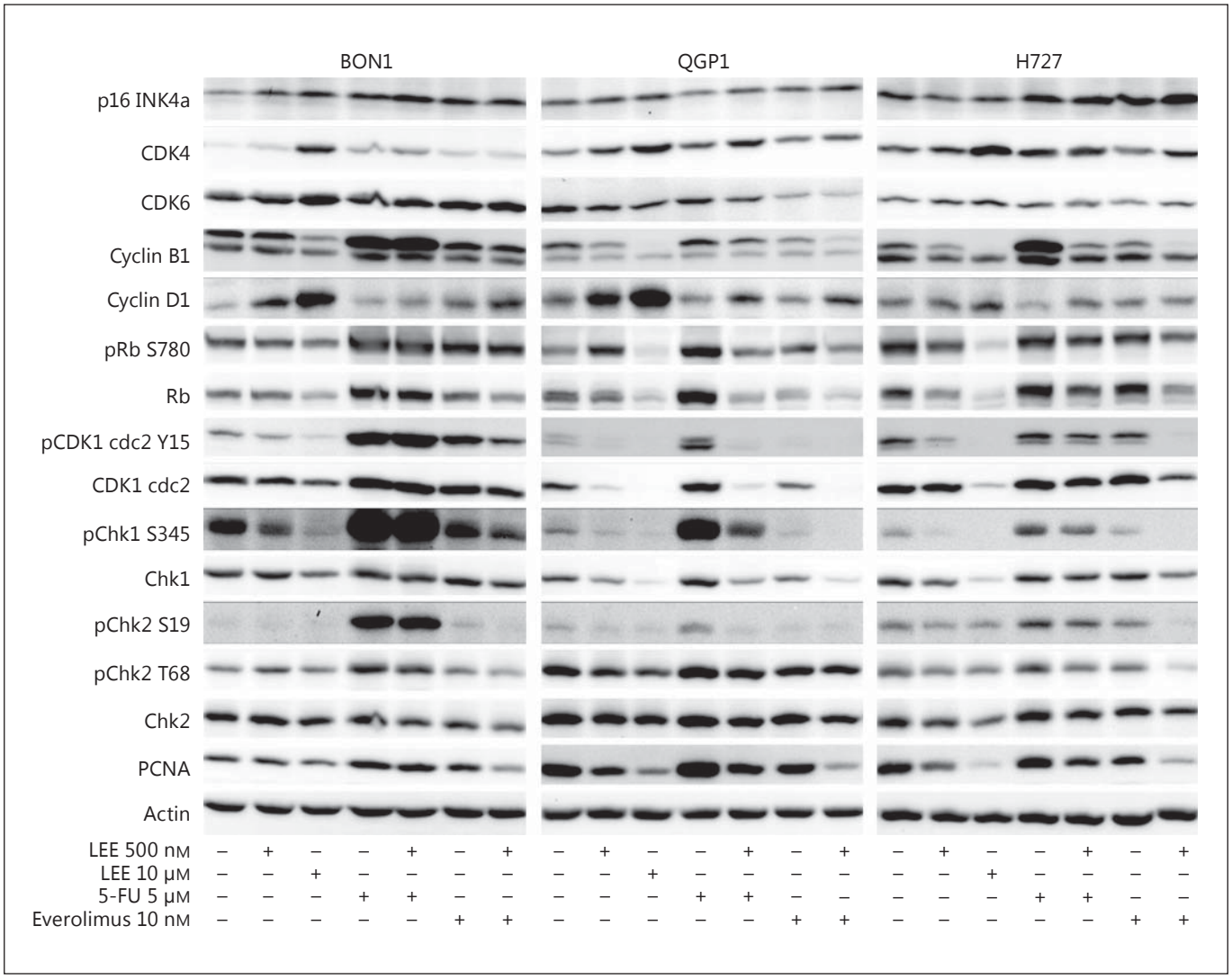

Fig. 9. The effects of LEE011 (500 nM) alone and in combination with 5-FU (5 μM) and everolimus (10 nM) on components of the cyclinD-CDK4/6-Rb axis, CDK1, and PCNA in BON1, QGP1, and H727 cells after 72 h of incubation analyzed via Western blot. A representative blot from 3 independently performed experiments is shown.

tagonized the unfavorable proproliferative phosphorylation of Akt, 4EBP1, and Erk when compared to the respective single-substance treatments. The treatment with LEE011 and everolimus, in particular, showed a strong effect on dephosphorylation of Erk in all 3 cell lines.

LEE011 Alone Downregulates the CyclinD-CDK4/6-Rb Axis and Combined Treatment with 5-FU or Everolimus Affects Cell Cycle-Regulating Components

Western blot analysis revealed the expression level of cell cycle-relevant protein components in human neuroendocrine pancreatic BON1, pancreatic islet QGP1 and bronchopulmonary NCI-H727 tumor cells when stimulated for 72 h with LEE011 (10 μM) alone and in combination treatment with LEE011 (500 nM) and 5-FU (5 μM) or everolimus (10 nM) (Fig. 9). In all 3 cell lines, the endogenous CDK4/6 inhibitor p16 is upregulated in the combination treatments with 5-FU and everolimus. In BON1 and QGP1 cells, p16 is also upregulated upon single LEE011 (10 μM) treatment. Possibly due to feedback mechanisms, CDK4 and CDK6 show an increase in expression with LEE011 (10 μM) treatment. LEE011 at a

concentration of 10 μM decreases the cyclin B1, pRb, Rb, pCDK1 and CDK1 levels and increases the cyclin D1 level. In QGP1 and NCI-H727 cells, the combination treatment with everolimus cooperatively downregulated cyclin B1, pRb, Rb, pCDK1, and CDK1. In BON1 cells, the combination treatment with everolimus only cooperatively downregulated Rb, but it attenuated the effects of everolimus on Cyclin B1, pCDK1 and CDK1. In all 3 cell lines, combined treatment with LEE011 and either 5-FU or everolimus attenuated the upregulation of cyclin D1. On the other hand, the 5-FU-mediated upregulation of cyclin B1, pRb, Rb, pCDK1, and CDK1 in QGP1 and NCI-H727 cells was attenuated by the combination treatment with LEE011 (500 nM). In all 3 cell lines, proliferating cell nuclear antigen (PCNA) was downregulated by either high concentrations of LEE011 (10 μM) alone or by combination treatment with everolimus (10 nM) and was attenuated in QGP1 and NCI-H727 cells treated with a combination of LEE011 (500 nM) and 5-FU (5 μM).

Discussion

Therapeutic approaches against GEP-NETs are of limited efficacy, primarily due to their highly heterogeneous characteristics and the late-stage detection [1, 3]; ergo, new strategies are urgently needed [4]. Nonspecific CDK inhibitors have been of limited efficacy in cancer treatment, and they have exhibited highly cytotoxic side effects [10]. The small molecule CDK4/6 inhibitor LEE011 has demonstrated antiproliferative characteristics with only mild side effects [10]. Therefore, in this in vitro study, we evaluated the novel and specific CDK 4/6 inhibitor LEE011 as a possible new molecular therapeutic strategy for NET treatment.

Our results suggest a relationship between the level of Rb and cyclin D1 expression and sensitivity to LEE011 treatment. The treatment-sensitive BON1, QGP1, and NCI-H727 cells showed elevated levels of Rb and cyclin D1 in comparison to LEE011 treatment-resistant GOT1 cells with barely detectable Rb and cyclin D1 levels (Fig. 4). Similar results were observed in an in vitro study with breast cancer cell lines, where CDK4/6 inhibitor (PD 0332991) treatment-sensitive cell lines also expressed a high cyclin D1 and Rb protein level [20]. A high expression level of the oncogene *Cyclin D1* has been shown to have a pathogenic role in parathyroid tumorigenesis and GEP-NETs [21]. Hence, we suggest that GEP-NET treatment with LEE011 has a high therapeutic relevance. In addition, high cyclin D1 expression levels were detected in a study that included 92 patients with pNETs [22]. While loss of Rb is the reason for an augmented cell cycle and proliferation in some cancer types, in most cancer types, Rb remains in its wild-type state, including in NETs [23–26]. Rb-proficient wild-type cell lines depend mostly on cyclinD-CDK4/6 regulation for proliferation and are likely to be susceptible to CDK4/6 inhibitory treatments [27, 28]. Typical carcinoids (100%) and atypical carcinoids (79%) of the lung exhibited Rb expression [24]. In our study, GOT1 cells showed only minimally detectable expression levels of Rb and cyclin D1 (Fig. 4). This stands in contrast to the clinical situation, as Rb and cyclin D1 were found to be highly expressed in various NET tumor samples [21, 22, 24]. A whole-exome comparison of primary well-differentiated NET samples versus BON, QGP1, and NCI-H727 cell lines showed substantial differences in mutation rates and mutation patterns [29, 30]. These data [29, 30] demonstrate that available human NET cell lines might not adequately reflect the genetic and biological entities of well-differentiated NETs. Therefore, the in vitro results of our study must be interpreted and extrapolated with caution with regard to the clinical situation. In a xenograft mouse model, tumor growth of the QGP1 cell line was also inhibited by the CDK4/6 inhibitor PD 0332991 [22]. Many studies also reported a low p16 expression level with high CDK4/6 inhibitor susceptibility [20, 28, 31]. Here, we show that, on the one side, even a moderate expression of p16 (in BON1, QGP1, and NCI-H727 cells) (Fig. 4) does not comprise the antitumor efficiency of LEE011 (Fig. 2), and on the other side, low p16 expression in GOT1 cells (Fig. 4) did not sensitize cells to LEE011 treatment (Fig. 2). CDK4/6 inhibition was suggested to be effective in tumor entities with CDK4 overexpression or amplification [10]. In mice, the development of pancreatic islet or pituitary MEN-1 tumorigenesis required a functional *Cdk4* gene [32]. Furthermore, Tang et al. [22], found an upregulation of CDK4 in pNETs, which led to subsequent phosphorylational inactivation of the retinoblastoma protein (Rb), and they detected a *Cdk4* or *Cdk6* copy number increase in 19% of the cases in a genetic analysis of 26 pNETs. In our cell lines, CDK4 was expressed in all cell lines, but the expression levels of CDK4 and CDK6 were not markers for LEE011 treatment sensitivity (Fig. 4). We suggest that the treatment resistance of GOT1 cells arises due to deficient Rb expression and low expression of cyclin D1 (Fig. 4), which indicates that the cyclinD-CDK4/6-Rb axis is neglectable for GOT1 cell proliferation [10, 27].

In neuroendocrine pancreatic BON1, pancreatic islet QGP1 and bronchopulmonary NCI-H727 cells, we ob-

served a time- and dose-dependent decrease in cellular survival with LEE011 treatment (Fig. 2) and found significant effects on NET cell cycle: A clear shift into G1 phase cell cycle arrest was observed in all 3 NET cell lines tested (Fig. 5). Similar results regarding a decrease in cell survival and G1 cell cycle arrest through inhibition of CDK4/6 with PD 0332991 were observed in breast cancer cell lines and ovarian cancer [20, 28]. Wu et al. [33] 2011 also observed G1 phase cell cycle arrest due to CDK4 knock down in different lung cancer cells. Unfortunately, LEE011 treatment caused a dose-dependent increase in the expression of oncogenic cyclin D1 and CDK4/6, possibly due to feedback loop mechanisms, which could lead to de novo treatment resistance (Fig. 9). In estrogen-positive breast cancer cells, similar feedback mechanisms regarding cyclin D1 were detected, which led to cytostasis evasion [34]. In a HER2-positive breast cancer mouse model, dual targeting prevailed against an acquired cyclin D1-CDK4 upregulation-mediated treatment resistance [35]. Therefore, combination treatment strategies are a rational option, not only to enhance single substance efficacy but also to overcome possible treatment resistances and feedback loops.

The chemotherapeutic agent 5-FU is a clinically applied drug that causes cytotoxic DNA damage and activates apoptosis [36]. 5-FU and capecitabine are commonly used in the treatment of pNETs in various regimens, such as STZ/-FU, Cap/TEM, FOLFOX, or FOLFIRI [37–39]. The combined treatment with LEE011 and 5-FU was a rational combination choice to maximize the antiproliferative efficacy, as the 2 drugs target different pathways. As a consequence, the combination treatment showed a significant enhancement in cellular growth decrease (Fig. 6). A similar outcome was shown in a study with human ovarian cancer cell lines, where the CDK4/6 inhibitor PD-0332991 enhanced the effects of chemotherapy [28]. In addition, the combination treatment downregulated oncogenic components of the PI3K-Akt-mTOR pathway and Ras-Raf-MEK-ERK pathway (Fig. 8). Many studies indicate the importance of the Ras-Raf-MEK-ERK and the PI3K-Akt-mTOR molecular signaling pathway for NET cell growth, invasion, and proliferation [16, 40–46]. In addition, 5-FU showed cooperative downregulating effects with LEE011 on cell cycle components such as oncogenic cyclin D1 (Fig. 9), which is often overexpressed in GEP-NETs and influences tumorigenesis and cell proliferation in NETs [21, 22]. Unfortunately, the ability of 5-FU to activate apoptotic mechanisms of cell death due to DNA damage [36] seems to be attenuated by LEE011, as evidenced by the downregulation of proapoptotic PARP cleavage (Fig. 7a) and caspase-3/7 activity (Fig. 7b). The DNA damage response induces 2 cell cycle checkpoint kinases (Chk1 and Chk2) at G2/M and G1/S phase transition, respectively [47]. LEE011 decreases the levels of Chk1/2 and their phosphorylation in NETs, and, as a result, the cell cycle is permanently arrested (Fig. 7c). Downregulation of Chk1 was also observed in a study with PD0332991 [48]. Although active Chk1 inhibition is correlated with an enhancement of 5-FU cytotoxicity [49], in combination with LEE011 the downregulation of Chk1/2 is due to the G1 phase cell cycle arrest rather than to specific Chk1-inhibiting effects, proving again the antagonistic effects of LEE011 + 5-FU; CDK4/6 inhibition causes cell cycle arrest without passing beyond the restriction point of Chk activation, hence abrogating the need for negative regulation of mitosis entry [50]. Given our data, we assume that the permanent G1 phase cell cycle arrest as a consequence to CDK4/6 inhibition by LEE011 might rescue the cells from undergoing apoptotic cell death in response to 5-FU treatment. Similar results are shown in a study with triple-negative breast cancer models, where CDK4/6 inhibition counteracted the cytotoxic response of doxorubicin, and in an in vivo study, where the DNA-damaging effects of carboplatin were also antagonized by the CDK4/6 inhibitor [48, 51, 52]. Hence, we recommend further investigation of the effects of 5-FU in combination with CDK4/6 inhibitors to clarify possible antagonizing effects, and we expect the ongoing phase 1 study with paclitaxel and the CDK4/6 inhibitor palbociclib to examine this convoluted matter in the clinic (NCT01320592).

The PI3K-Akt-mTOR pathway is an important and often constitutively activated pathway in NETs [16, 46]. The mTOR inhibitor everolimus has been investigated in NETs in several clinical phase 3 trials [53]. Everolimus has been approved for the treatment of advanced pNETs [54–57] and for the treatment of gastrointestinal NETs and lung NETs [53], and it is one of the established treatment options according to ENETS guidelines [53]. Single-substance treatment with everolimus has been shown to lead to resistance mechanisms in many tumor entities, including pNETs [58–61]. Dual-targeted therapy approaches to overcome possible resistance mechanisms and feedback loops have shown promising results in NETs [40, 62]. In PI3K inhibitor-resistant cancer cells, combined treatment with a CDK4/6 inhibitor re-sensitized cancer cells to PI3K inhibition [63]. The PI3K-Akt-mTOR pathway is also a crucial molecular signaling pathway in promoting cyclinD-CDK4/6-dependent proliferation, as it converges into the cell cycle pathway at that

point [64]. Combined treatment with the mTORC1-downregulating everolimus significantly enhanced the decrease in cellular survival in all NET cell lines tested (Fig. 6). Similar effects on cell survival were obtained in a study with breast cancer, where the combination of a CDK4/6 inhibitor with a PI3K inhibitor synergistically inhibited tumor cell viability [63]. Additionally, the combination treatment with everolimus downregulated the oncogenic PI3K-Akt-mTOR pathway and the Ras-Raf-MEK-ERK pathway (Fig. 8) and showed cooperative effects on the downregulation of cell cycle components (Fig. 9). Similar to the combination treatment with 5-FU, the combination of LEE011 and everolimus also showed cooperative effects in blocking oncogenic cyclin D1 (Fig. 9) [21, 22]. In addition, the endogenous CDK4/6 inhibitor p16 is upregulated upon combined LEE011 and everolimus treatment in all 3 cell lines (Fig. 9), supporting the exogenous CDK4/6 inhibition of LEE011. Furthermore, the combination treatment with everolimus and LEE011 showed no crucial antagonizing effects on apoptotic cell death mechanisms (Fig. 7). mTOR activity has been shown to have little association with apoptotic signalling mechanisms, and only high doses of everolimus could induce apoptotic cell death mechanisms in NETs [16, 65]. In addition, the combination treatment (LEE011 + everolimus)-mediated decrease in Chk expression and phosphorylation (Fig. 7c) had no appreciable impact on treatment efficiency, as everolimus is a molecular targeting substance, not a DNA damaging agent [66]. Furthermore, the combination treatment with everolimus showed strong agonistic effects with LEE011 in PCNA inhibition (Fig. 9). PCNA is a DNA polymerase accessory protein that is implicated in different cellular processes, such as DNA replication, DNA repair, and cell cycle control [67]. PCNA has been found to actively bind to some Cyclins and their corresponding kinases to support cell cycle progression and DNA replication [67, 68]. Inhibition of PCNA due to either endogenous p21 or exogenous inhibitors leads to immediate cell cycle arrest [69, 70]. Interestingly, everolimus was shown to increase G1 phase cell cycle arrest in NET cells, assuming a possible reinforcing effect for combination with CDK4/6 inhibitors such as LEE011 [16]. Current clinical phase 1b studies are investigating the combination of LEE011 and everolimus in patients with breast cancer (NCT01857193 and NCT02732119). Taken together, our data regarding the combination treatment with LEE011 and everolimus in NETs in vitro suggest that this dual-targeting strategy may be an effective regimen for a novel therapeutic strategy against NETs.

Conclusion

We demonstrated that the highly selective CDK4/6 inhibitor LEE011 exerts significant antitumoral efficacy in NET cell lines in vitro, either alone or in a dual-targeting approach together with 5-fluorouracil or everolimus. The small molecule CDK4/6 inhibitor LEE011 effectively blocks cellular proliferation through downregulation of the cyclinD-CDK4/6-Rb axis. Thus, CDK4/6 inhibition with LEE011 might be an effective new therapeutic regimen for NETs. Currently, a clinical phase 2 trial with LEE011 is recruiting patients with advanced NETs of foregut origin (NCT02420691), and another clinical phase 2 trial is recruiting patients with CDK4/6 pathway-activated tumors (NCT02187783).

Acknowledgements

This work contains parts of the unpublished doctoral thesis of E.T. Aristizabal Prada. The manuscript was edited for proper English diction, grammar, punctuation, spelling, and overall style by native English-speaking editors at American Journal Experts.

Disclosure Statement

C.J. Auernhammer has received research contracts (Ipsen, Novartis), lecture honorarium (Ipsen, Novartis, Pfizer, Amgen, Roche, Falk), and advisory board honorarium (Novartis). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Funding Sources

E.T. Aristizabal Prada received a scholarship from FAZIT-Stiftung (http://www.fazit-stiftung.de). This study was funded by an unrestricted research grant from Novartis Pharma GmbH, Nürnberg, Germany.

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